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• GASTRIC CANCER •

Helicobacter pylori promote gastric cancer cells invasion through a NF-κB and COX-2-mediated pathway

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Abstract

AIM: To examine the effects of *Helicobacter pylori* (*H pylori*) infection on the invasiveness of gastric cancer cells, and to elucidate its mechanism.

METHODS: Gastric carcinoma cells, MKN-45, were incubated with CagA-positive *H pylori*, and cell invasion was determined by Matrigel analysis. The expression of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) were assessed by Western-blot analysis, and transcriptional activation of the COX-2 promoter was examined by measuring luciferase and β-galactosidase activities. Lastly, the protein-DNA interaction was confirmed by an electrophoretic mobility shift assay.

RESULTS: The current studies showed that: (1) incubation of CagA-positive *H pylori* with MKN-45 cells significantly promotes gastric cancer cells invasion, and this effect is attenuated by pre-treatment with NS-398, a COX-2 inhibitor, or PDTC, a nuclear factor κB (NF-κB) inhibitor; (2) the induction of MKN-45 cells invasion by *H pylori* is associated with increases in COX-2, MMP-9, and VEGF protein expression, and co-incubation of NS-398 or PDTC significantly reduces these effects; (3) *H pylori* infection transactivates COX-2 promoter activity and increases the binding of NF-κB to this promoter.

CONCLUSION: Our data demonstrate that *H pylori* infection promotes gastric epithelial cells invasion by activating MMP-9 and VEGF expression. These effects appear to be mediated through a NF-κB and COX-2 mediated pathway, as COX-2 or NF-κB inhibitor significantly attenuate the invasiveness of gastric cancer cells and the expressions of MMP-9 and VEGF protein.

Key words: *H pylori*; Gastric cancer; Invasion; MMP-9; VEGF; COX-2; NF-κB

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a spiral, microaerophilic, neutralophilic gram-negative bacterium that colonizes the gastric mucosa in 25-50% and 70-90% of the population in the developed and developing countries, respectively[1]. *H pylori* is believed to be the major contributing factor to the development of chronic gastritis and peptic ulcer diseases in human, and epidemiological and interventional studies in human as well as in experimental animals strongly suggest that *H pylori* infection increases the risk of adenocarcinoma in the distal stomach[2,3]. Although *H pylori* has been demonstrated to be associated with gastric cancer occurrence, whether *H pylori* promote gastric cancer cells invasion is still unknown.

Upon bacterial infection, host effectors induced by *H pylori* are likely to contribute to gastric carcinogenesis and tumor invasion. Matrix metalloproteinases (MMPs), a family of closely related enzymes that degrade extracellular matrix (ECM), are considered to be important factors in facilitating tumor invasion and spread[4]. MMPs displays broad and overlapping substrate specificity and collectively and they are capable of degrading the major components of ECM. Furthermore, MMPs are found to play major roles in connective tissue remodeling during pathologic conditions, such as cancer and inflammatory disease. Among these MMPs,

matrix metalloproteinase-9 (MMP-9) has been considered to be an important factor in facilitating lymphatic invasion and metastases in early gastric carcinoma[5], and the level of tissue MMP-9 has been shown to be related to the overall survival of patients with gastric carcinoma[6]. Recently, MMP-9 has been reported to be induced by *H pylori* through activation of nuclear factor κB (NF-κB)[7]. Whether *H pylori* can promote gastric cancer cell invasion through MMP-9 is unknown.

Vascular endothelial growth factor (VEGF), the most well-characterized angiogenic factor, is known to play a major role in the multistep process leading to the reconstruction of normal mucosa architecture. This process is believed to be mediated through angiogenesis, ensuring an adequate supply of nutrients to the healing tissue[8]. Moreover, VEGF also plays a vital role in tumor-associated microvascular invasion[9]. In human gastric cancers, VEGF has been found to be over-expressed[10,11], and in a recent study, VEGF expression has been reported to be upregulated by *H pylori* through a cyclooxygenase-2 (COX-2) dependent mechanism [12]. Whether VEGF contributes to gastric cancer invasion induced by *H pylori* infection remains unknown.

Cyclooxygenase (COX), the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin H_2, is the main target of non-steroid anti-inflammatory drugs (NSAIDs). Two isoforms of this enzyme have been identified: COX-1 is constitutively expressed in most tissues and is involved in the production of prostaglandins to maintain normal physiological functions; and COX-2 is involved in inflammation and has been shown to be induced by mitogens, cytokines, hormones, and growth factors. Several recent studies suggested that COX-2 might be an important factor in carcinogenesis, and COX-2 inhibitors were shown to possess anticancer effects. These properties were mediated through the inhibition of prostaglandins production by COX-2, leading to decreases in angiogenic factors, and changes in MMP activity[13]. In human gastric cancer cells, NF-κB mediated COX-2 expression is associated with cell proliferation[14]. Furthermore, *H pylori* activates NF-κB expression in gastric cancer cells[15].

The present study was undertaken to examine the effect of *H pylori* infection on gastric cancer cells invasiveness and to elucidate its mechanism. Our results suggest that *H pylori* may induce the expression of MMP-9 and VEGF and promote gastric cell invasion through a NF-κB-and COX-2-mediated pathway.

MATERIALS AND METHODS

Cell line

Human gastric carcinoma cell line, MKN-45, was obtained from American Type Culture Collection (Manassas, VA, USA). MKN-45 cells were maintained in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. On the day of experiment, cells were refed with fresh medium and co-cultured with *H pylori*.

Bacterial strain

Cag pathogenicity island-positive *H pylori* (ATCC 43504) strain was used in experiments described in this study. Stock cultures were maintained at -70 ℃ in brucella broth supplemented with 30% glycerol. The bacteria were grown at 37 ℃ in 5% horse blood agar plates and in a microaerobic condition. Cultures were routinely screened for urease activity. For co-infection studies, *H pylori* were harvested between 48 and 72 h after inoculation of agar plates, resuspended in sterile phosphate buffered saline (PBS), and enumerated by absorbance at 600 nm (1 optical density (A) at 600 nm = 2.4×10^8 colony-forming units/mL). MKN-45 cells were seeded into 65 mm dishes, and *H pylori* at a multiplicity of infection 80 were added in the culture.

Cell migration assays

The effect of *H pylori* infection on cell migration was examined using the Matrigel Invasion chamber, as suggested by the manufacturer (BD Bioscience). The lower surface of the chamber contained a transwell filter (8-μm pores), coated with fibronectin, and vitronectin. MKN 45 cells (1×10^5) and *H pylori* were added to the upper chamber, in the presence or absence of NS-398, and incubated overnight in a humidified tissue culture incubator, at 37 ℃, 50 mL/L CO_2 atmosphere. The next day, a cotton tipped swab was inserted into chambers to remove non-invading cells by applying gentle but firm pressure while moving the tip around the membrane surface. The cells on the lower surface of insert chambers were stained with hematoxylin for 10 min, and the cell number was counted under a microscope (40 to 200× magnifications). The extent of cell invasion was expressed as fold increases of total number of cells on the lower surface of chambers in treated over untreated samples.

Western blot analysis

To obtain whole-cell extracts, cells were washed twice with ice-cold phosphate-buffer saline (PBS), and pelleted by centrifugation (200 r/min). Cell pellets were then lysed in a standard RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), containing protease inhibitors. Protein concentrations were determined by Bio-Rad assays. Protein samples were dissolved in the loading buffer (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 100 mmol/L dithiothreitol, and 0.01% bromophenol blue), heated to 100 ℃ for 3 min, and loaded onto the gel in an electrophoresis buffer containing 25 mmol/L Tris-HCl, pH 8.3, 250 mmol/L glycine, and 0.1% SDS. At the completion of electrophoresis, protein was transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Science). The membrane was incubated in the blocking buffer (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20), containing 5% nonfat powdered milk for 2 h. The membrane was immunoblotted with COX-2, MMP-9, or VEGF antiserum (obtained from Santa Cruz Biotech, Santa Cruz, CA, USA). After incubation with the secondary antibody, the membrane was visualized with Enhanced Chemiluminescence kit from Amersham.

Transfection and luciferase assay

To examine transcriptional regulation of COX-2 promoters by NF-κB, MKN-45 cells were transiently transfected with pMT2-LacZ and COX-2-Luc or phPES2 (KBM)-Luc DNAs, in the presence of NF-κB p65, p50, or control pMT2 plasmid (kindly supplied by Dr. Gail Sonenshein, Boston Medical

Center, Boston, MA, USA). The phPES2 (KBM) plasmid contains a full-length COX-2 promoter in which a putative NF-κB binding site is mutated, as described previously[16]. For luciferase assays, cells were washed twice with PBS and then lysed in 500 μL of lysis buffer following the manufacturer's instructions (Analytical Luminescence, San Diego, CA, USA). To assay luciferase activity, cell lysate (100 μL) was mixed with 100 μL of luciferase substrate solution A (Analytical Luminescence). Using a luminometer with automatic injection, 100 μL of luciferase solution B was added (Analytical Luminescence) and luciferase activity was detected as the light emission over a 30-s period.

The β-galactosidase activity in 40 μL of the cell lysate was determined after a 5-30-min incubation at 37 ℃ with 2 mmol/L chlorophenol red β-galactopyranoside (Boehringer Mannheim) in 2 mmol/L $MgCl_2$, 0.1 mmol/L $MnCl_2$, 45 mmol/L 2-mercaptoethanol, and 100 mmol/L $NaHPO_4$, pH 8.0. The reactions were terminated by adding 500 μL of 0.5 mol/L EDTA, pH 8.0, and the absorbance at 570 nm was measured using a spectrophotometer. With each experiment, the luciferase activity was determined in duplicate and normalized to the β-galactosidase activity for each dish.

Electrophoretic mobility shift assay

Nuclear extracts were prepared using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). A double-strand oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'), corresponding to the putative NF-κB binding domain on the COX-2 promoter was synthesized and was labeled with [γ-^{32}P] ATP (3 000 Ci/mmol at 10 mCi/mL) using a T4 polynucleotide kinase. Nuclear protein (1 μg) was incubated in a buffer containing 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25 mg/mL poly (dl-dC)·poly (dl-dC), for 10 min at room temperature, and ^{32}P-labeled NF-κB oligo was added to each reaction and incubated for additional 20 min. Samples were subjected to electrophoresis at room temperature on a 4% acrylamide gel at 25 mA using 0.5× TBE buffer. The gels were dried at 80 ℃ for 2 h and exposed to radiography film at -70 ℃.

Immunohistochemical studies

To further investigate the role of MMP-9 and VEGF in gastric cancer invasion, the expression of these two proteins were examined in *H pylori*-positive gastric cancer tissues. Tissue sections from gastric cancer were de-paraffinized, rehydrated, and immersed in 3% hydrogen peroxide–methanol solution for 10 min at room temperature to inhibit endogenous peroxidase activity. The sections were then incubated with unmasking solution (0.01 mol/L citrate buffer pH 6.0), heated for 10 min. The sections were allowed to cool down to room temperature and washed twice in PBS buffer for 5 min. The sections were pre-incubated with diluted normal serum for 10 min, and then incubated with 1:40 mouse anti-MMP-9 monoclonal antibody (Novocastra Lab. Ltd, Newcastle, UK), 1:100 rabbit anti-VEGF polyclonal antibody (Zymed Lab. Inc., South San Francisco, CA, USA), or 1:40 mouse anti-COX-2 monoclonal antibody (Cayman Chemical Co., Ann Arbor, MI, USA) for 1 h at room temperature. The sections were washed in PBS for 5 min twice, and incubated with appropriate biotinylated secondary antibody. After washing in PBS for 5 min twice, the slides were incubated with ABC reagents, followed with DAB or other suitable peroxidase substrates. The sections were counterstained with hematoxylin for 30 s, washed, dried, and mounted.

RESULTS

H pylori infection promotes gastric cancer cells invasion

In vivo tumor invasion includes not only migration process, but also adhesion, proliferation and angiogenesis, *etc.*, dissociation of *in vitro* assay for tumor cell invasion (migration) with *in vivo* tumor invasion might exist. However, similar migration assay to assess tumor cell invasion has been used by several articles published recently[17].

We used Matrigel invasion chamber to examine whether *H pylori* infection induces gastric cancer cells invasion and to determine the effect of COX-2 inhibitor on this process. MKN-45 cells were incubated with *H pylori* in the presence or absence of COX-2 inhibitor, NS-398 (100 ng/mL). As illustrated in Figure 1A co-infection with *H pylori* induced a 2.5 fold increase in MKN-45 cells migrated through Matrigel-coated filters, indicating that *H pylori* promoted gastric cancer cells invasion. The COX-2 inhibitor, NS398, significantly reduced cell invasion in *H pylori* stimulated, but not in untreated cells. This result suggested that *H pylori* induced gastric cancer cells invasion was, in part, mediated through a COX-2-dependent mechanism.

To examine the effect of NF-κB on the migration of MKN-45 cells, cells were incubated with *H pylori* in the presence or absence of NF-κB inhibitor, (pyrrolidine dithiocarbamate (PDTC), 0.1 μmol/L, purchased from Sigma Chem, St. Louis, MO, USA). As illustrated in Figure 1B the effect of *H pylori* infection on gastric cancer cells migration was completely abolished by NF-κB inhibitor, suggesting a potential involvement NF-κB in this process. The representative microscopic photos of stained cells treated with *H pylori* in the presence or absence of COX-2 inhibitor were shown in Figures 1C-E.

H pylori infection induces MMP-9 and VEGF expression

Several proteins, including MMP-9 and VEGF, have been reported to play an important role in tumor invasion. The effects of *H pylori* on the expression level of MMP-9 and VEGF were examined in MKN 45 cells after co-infection with *H pylori* for a different period of time. The induction of VEGF protein expression was noticeable within an hour after *H pylori* infection and reached the highest level in 24 h (Figure 2A). Although the increment of MMP-9 level after *H pylori* infection was smaller than that of VEGF, a significant increase was observed at 24 h (Figure 2A).

COX-2 and NF-κB inhibitors reduce MMP-9 and VEGF expressions

Recent studies showed that COX-2 inhibitor reduced the release of MMP and COX-2-induced MMP-9 expression[18,19]. Therefore, we examined whether the activation of MMP-9 and VEGF by *H pylori* infection was also dependent on COX-2 expression. The MKN-45 cells were co-cultured

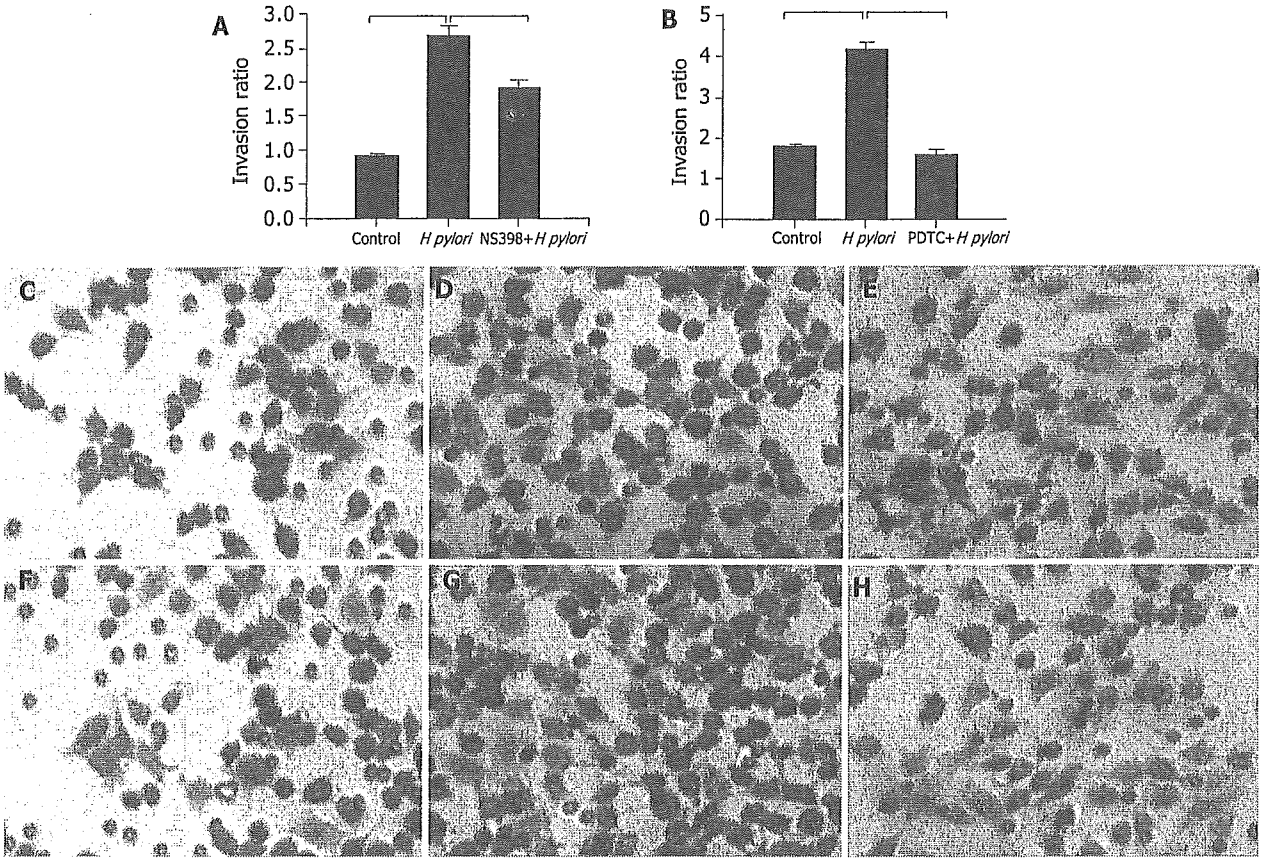

Figure 1 Effects of *H pylori* infection, a cox-2 (NS398), or a NF-κB inhibitor (PDTC) on gastric cancer cell invasion. **A:** MKN-45 cells were treated with *H pylori* in the presence or absence of NS398. Cells on the lower surface of insert chamber were stained with hematoxylin for 10 min and counted under microscope with 200× magnifications. Data are presented as mean±SD of three separate experiments ($P<0.05$); **B:** MKN-45 cells were treated with *H pylori* in the presence or absence of PDTC ($P<0.05$); microscopic photos of stained migration cells: **C:** control; **D:** with *H pylori*; **E:** with *H pylori* and NS-398; **F:** control; **G:** with *H pylori*; **H:** with *H pylori* and PDTC.

with *H pylori* for 24 h in the presence or absence of a COX-2 inhibitor, NS-398, and our results showed that co-culture with *H pylori* resulted in a time-dependent increase in COX-2 protein concentration in MKN-45 cells (Figure 2B). Moreover, NS398 significantly reduced the expression levels of COX-2, MMP-9, and VEGF, induced by *H pylori* in MKN-45 cells at 24 h (Figure 3A). These data suggest that the induction of MMP-9 and VEGF by *H pylori* is mediated through a COX-2-dependent mechanism.

To examine whether activations of MMP-9 and VEGF by *H pylori* infection were also dependent on NF-κB expression, MKN-45 cells were co-cultured with *H pylori* for 24 h in the presence or absence of a NF-κB inhibitor, PDTC. As demonstrated in Figure 3B, the effects of *H pylori* on COX-2, MMP-9, and VEGF expression were significantly reduced by PDTC. These results suggest that the induction of COX-2, MMP-9, and VEGF by *H pylori* is also NF-κB-dependent.

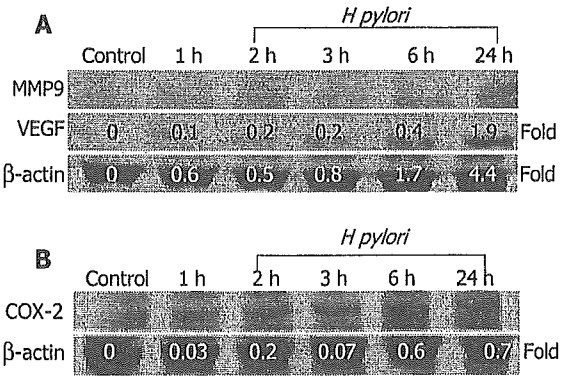

Figure 2 *H pylori* infection increases MMP-9, VEGF, and COX-2 expressions in gastric epithelial cell. MKN-45 gastric cancer cells were incubated with *H pylori* for 0-24 h and total cellular protein was extracted for Western blot analysis for the expression of **A:** MMP-9 and VEGF and **B:** COX-2 proteins. The blots were stripped and probed with β-actin to document equal protein loading. The experiment was performed for thrice with similar results.

Induction of MMP-9 and VEGF by H pylori infection depends on a NF-κB-mediated COX-2 activation

A previous study has shown that NF-κB regulated COX-2 expression and affected cell proliferation in human gastric cancer cells[14]. We hypothesize that the induction of MMP-9 and VEGF by *H pylori* is associated with NF-κB mediated COX-2 expression. To investigate this hypothesis, we first examined the effect of NF-κB on COX-2 promoter activities in MKN45 cells. As illustrated in Figure 4A co-transfection with NF-κB p65 or p50 plasmid DNA significantly enhanced COX-2 promoter activity, and the induction was completely

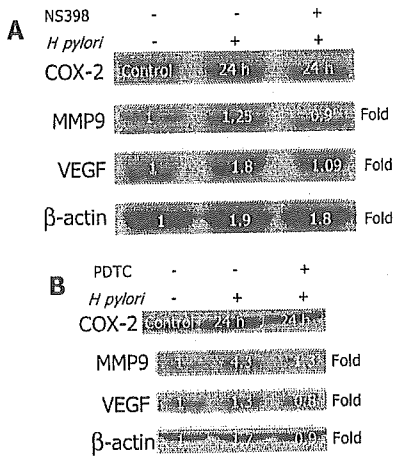

Figure 3 Effect of COX-2 or NF-κB inhibitor on COX-2, MMP-9, or VEGF protein level in MKN-45 cells. **A:** Cells were cultured in the presence or absence of *H pylori* and NS398 for 24 h, and cellular protein was extracted and subjected to Western blot analysis; **B:** MKN-45 cells were treated with or without PDTC and in the presence or absence of *H pylori* for 24 h. The experiments were repeated on at least three occasions, and the results were identical to these presented here. All blots were stripped and probed with β-actin to check for equal protein loading.

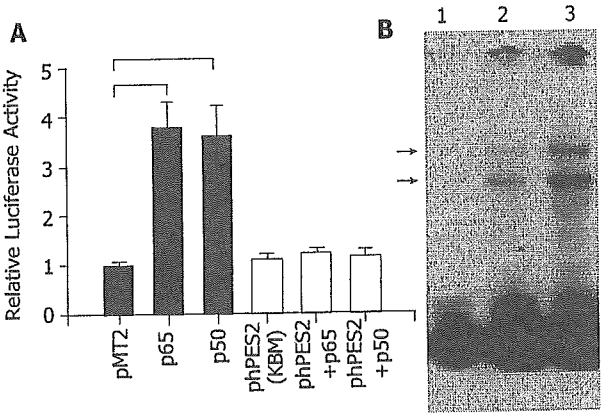

Figure 4 **A:** Transactivation of COX-2 promoter by NF-κB. MKN-45 cells were transiently transfected with full-length COX-2 promoter (Black bar), or mutated COX-2 construct, phPES2 (KBM), where the putative NF-κB binding domain was mutated (open bar), in the presence of p65, p50, or control vector (pMT2) plasmid. Luciferase and β-galactosidase activities were performed 48 h after transfection. ($n = 4$, $P<0.05$); **B:** Binding of nuclear NF-κB to COX-2 promoter DNA in MKN-45 cells. Nuclear protein was extracted from cells cultured in the absence (lane 2), or presence (lane 3) of *H pylori*. The protein-DNA interaction bands were shown as (→). Lane 1 showed free probe only.

abolished in phPES2 (KBM) construct where a putative NF-κB binding domain was mutated. Furthermore, the interaction between NF-κB and COX-2 promoter was also enhanced by *H pylori* infection in MKN-45 cells (Figure 4B).

High level of MMP-9 and VEGF expression in gastric cancer tissues with H pylori infection

To investigate whether these observations were also present *in vivo*, we randomly selected six gastric cancer patients (three cases with *H pylori* infection, confirmed by Giemsa stain and CLO test; three cases without *H pylori* infection) and examined the expression of COX-2, MMP-9, or VEGF protein in the surgical specimens. The immunostain of COX-2 (Figures 5A and B), MMP-9 (Figures 5C and D), or VEGF (Figures 5E and F) was located predominantly on the surface epithelial cells, and the intensity was more abundant in *H pylori*-positive than *H pylori*-negative tissue samples (Figure 5).

DISCUSSION

Gastric cancer is one of the most common malignancies in the world, especially in Eastern Asia. Although the incidence of gastric carcinoma is declining in Western countries, gastric

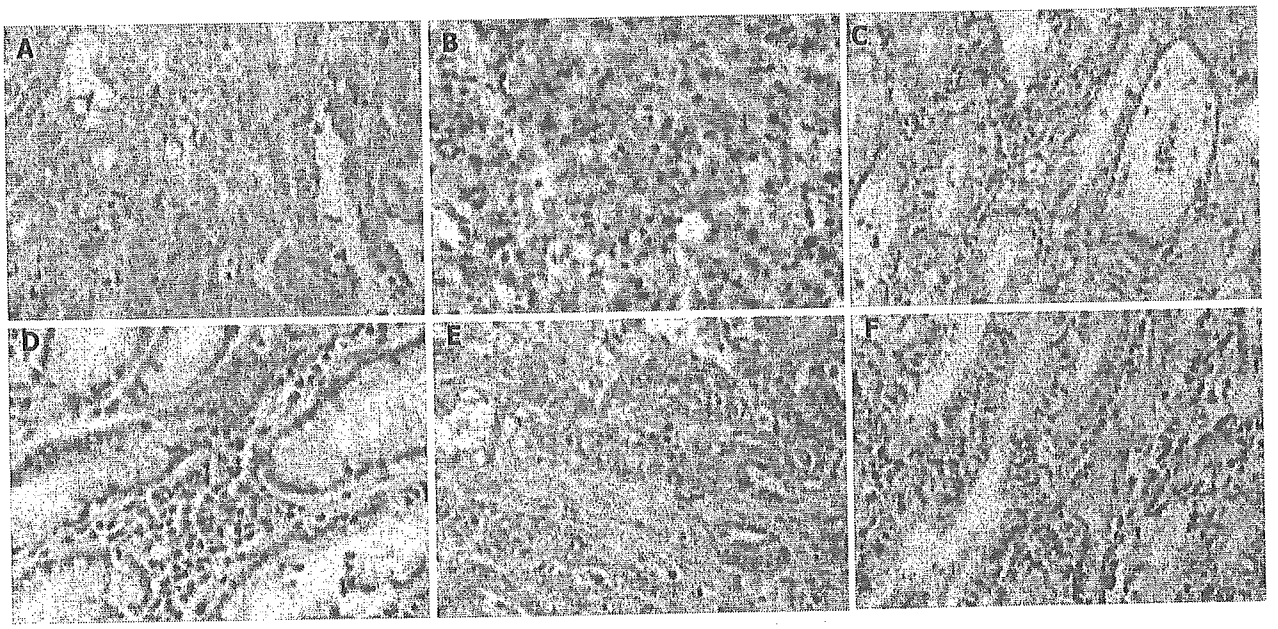

Figure 5 Immunohistochemical detection of COX-2, MMP-9, and VEGF in gastric cancer tissues. The serial sections of gastric cancer surgical specimens were stained with anti-COX-2 (**A** and **B**), anti-MMP-9 (**C** and **D**) , and anti-VEGF (**E** and **F**) antibodies. Sections were counterstained with hematoxylin. The immunoreactivities of COX-2, MMP-9 and VEGF are predominantly detected in the cytoplasm of the tumor cell. Tissues in A, C, and E are from *H pylori*-positive patients, whereas, sections in B, D, and F are from *H pylori*-negative individuals. (Magnification: **A-F**: 400×).

cancer remains the leading cause of cancer death worldwide. Current strategies to reduce mortality from this disease focus on early detection of gastric cancer or its precursor lesions by endoscopic screening. There is an increasing interest in the use of drugs to prevent the occurrence or the invasion of gastric cancers. Epidemiological studies have shown that NSAIDs decrease the risk of gastrointestinal carcinomas[20]. However, the mechanisms by which NSAIDs inhibit neoplastic growth are not fully elucidated[21,22].

The involvement of COX-2 in carcinogenesis has been shown in many epidemiological, animal and clinical studies. Individuals who took NSAIDs regularly had a markedly reduced risk of developing colon cancer[13], and COX-2 inhibitors have been proved to be effective in suppressing tumor progression both *in vitro* and *in vivo* in nude mice[21]. The anti-tumor effect of COX-2 inhibitors was attributed to their ability to induce apoptosis and inhibit tumor cell proliferation and angiogenesis. COX-2 inhibitors have also been illustrated to prevent tumor invasion in colon cancer, hepatocellular carcinoma[22], and lung cancer[23]. There is little evidence that COX-2 inhibitors may prevent gastric cancer invasion, although gastric cancers have been shown to over-express COX-2 protein[24].

In the present study, we found that *H pylori* infection promoted gastric cancer cells invasion and a COX-2 specific inhibitor significantly attenuated this process. Furthermore, the induction of gastric cancer cells invasion is associated with an increase in COX-2, MMP-9, or VEGF protein level, and these effects were also attenuated by a COX-2 inhibitor, suggesting a potential role of MMP-9 or VEGF in this process. In MKN-45 cells, *H pylori* infection enhanced nuclear NF-κB activity and transactivated COX-2 promoter. In addition, the induction of MMP-9 and VEGF by *H pylori* was suppressed by a NF-κB inhibitor. These data indicate that *H pylori*-induced MMP-9 and VEGF expressions in MKN-45 cells are mediated through the interaction of NF-κB on the COX-2 promoter. These results also support an important role of COX-2 in gastric cancer cells invasion.

Lim *et al.*, have recently shown that inhibition of NF-κB results in inhibition of COX-2 expression and proliferation of gastric cancer cells[14]. These data suggest NF-κB may play an important role in gastric cancer proliferation via COX-2 expression. Recently, Callejas *et al.*, reported that COX-2 expression promotes the release of MMP-9 in fetal rat hepatocytes[17], and Caputo *et al.*, also revealed that *H pylori* induce VEGF expression in MKN-28 gastric epithelial cells through a COX-2 dependent mechanism[12]. Furthermore, Li *et al.*, reported that COX-2 increased the angiogenic and metastatic potential of tumor cells by activation of VEGF in human transitional cell carcinoma cell line, and the effect on invasiveness could be reversed by COX-2 inhibitors[25-27].

Compared with previous studies, we have found several interesting points. In Figure 1 PDTC attenuated cell invasion completely, NS-398 only partially inhibited, suggesting that NF-κB could induce cell invasion not only through COX-2, but also through other pathways. One of the possibilities was through direct activation of VEGF expression. In Figure 3 we found that the inhibition of COX-2 by NS-398 attenuated MMP-9 expression to the control level, but it did not attenuate VEGF expression completely. On the other hand, the inhibition of NF-κB by PDTC inhibited both MMP-9 and VEGF expression to the control levels. These results suggest that MMP-9 expression is dependent on COX-2 pathway, while VEGF expression might be independent of COX-2 pathway. These observations might explain why NS-398 only partially attenuated cell invasion.

The activation of NF-κB by *H pylori* has been described by several groups[15,28,29]. Mori *et al.*, reported that *H pylori* induced NF-κB activation and stimulated MMP-9 expression[7]. In the present study, we have observed that *H pylori* infection in gastric cancer cells induced MMP-9 protein level and the increase was attenuated by either a NF-κB inhibitor or a COX-2 inhibitor. These data suggested that the expression of MMP-9 in *H pylori*-infected cells is mediated by a direct activation NF-κB, or through a COX-2 mediated pathway. This conclusion is supported by a recent report showing that MMP-9 promoter contains several putative NF-κB binding sites, and its transcription requires the activation of NF-κB.

Infection with *H pylori* affects more than 50% of the world population; some patients exhibit a progression through chronic atrophic gastritis to cancer, others develop peptic ulcer, but most do not exhibit either disease[30]. It is believed that different pathogens, host and environmental factors may lead to variable outcomes. In this study, we suggest that the induction of MMP-9 and VEGF proteins by *H pylori* can be considered part of a host response to accelerate an oncogenic progression via disruption of epithelial organization or increased invasion. The identification of *H pylori*-specific signaling pathways leading to the gastric cancer cells invasion will add to our understanding of the mechanism of *H pylori*-associated gastric carcinogenesis and the potential use of therapeutic agents in preventing *H pylori*-associated gastric cancer.

In summary, we have demonstrated that *H pylori* promote gastric epithelial cells invasion by activating the expression of MMP-9 and VEGF, and these effects are attenuated by a COX-2 or a NF-κB inhibitor. Moreover, *H pylori* infection induces nuclear NF-κB binding activity to the COX-2 promoter, and the activation of COX-2 promoter is abolished when the NF-κB binding site is mutated. These data suggest that the promotion of gastric cancer cells invasion by *H pylori* infection appears to be mediated through a NF-κB and COX-2 mediated pathway. Therefore, we proposed a model of *H pylori*-induced gastric cancer cell invasion as shown in Figure 6.

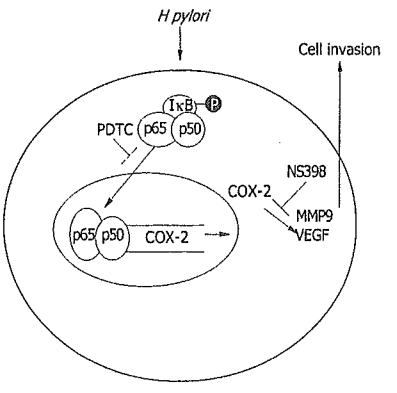

Figure 6 The schematic presentation of proposed mechanism of *H pylori* promote gastric epithelial cells invasion.

REFERENCES

1 Gooz M, Gooz P, Smolka AJ. Epithelial and bacterial metalloproteinases and their inhibitors in *H pylori* infection of human gastric cells. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G823-G832
2 **Huang JQ**, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. *Gastroenterology* 1998; **114**: 1169-1179
3 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789
4 **Murray GI**, Duncan ME, O'Neil P, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. *Nat Med* 1996; **2**: 461-462
5 **Kabashima A**, Maehara Y, Kakeji Y, Baba H, Koga T, Sugimachi K. Clinicopathological features and overexpression of matrix metalloproteinases in intramucosal gastric carcinoma with lymph node metastasis. *Clin Cancer Res* 2000; **6**: 3581-3584
6 **Sier CF**, Kubben FJ, Ganesh S, Heerding MM, Griffioen G, Hanemaaijer R, van Krieken JH, Lamers CB, Verspaget HW. Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma. *Br J Cancer* 1996; **74**: 413-417
7 **Mori N**, Sato H, Hayashibara T, Senba M, Geleziunas R, Wada A, Hirayama T, Yamamoto N. *Helicobacter pylori* induces matrix metalloproteinase-9 through activation of nuclear factor kappaB. *Gastroenterology* 2003; **124**: 983-992
8 **Jones MK**, Tomikawa M, Mohajer B, Tarnawski AS. Gastrointestinal mucosal regeneration: role of growth factors. *Front Biosci* 1999; **4**: D303-D309
9 **Connolly DT**, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989; **84**: 1470-1478
10 **Tian X**, Song S, Wu J, Meng L, Dong Z, Shou C. Vascular endothelial growth factor: acting as an autocrine growth factor for human gastric adenocarcinoma cell MGC803. *Biochem Biophys Res Commun* 2001; **286**: 505-512
11 **Yamamoto S**, Yasui W, Kitadai Y, Yokozaki H, Haruma K, Kajiyama G, Tahara E. Expression of vascular endothelial growth factor in human gastric carcinomas. *Pathol Int* 1998; **48**: 499-506
12 **Caputo R**, Tuccillo C, Manzo BA, Zarrilli R, Tortora G, Blanco CV, Ricci V, Ciardiello F, Romano M. *Helicobacter pylori* VacA Toxin Up-Regulates Vascular Endothelial Growth Factor Expression in MKN 28 Gastric Cells through an Epidermal Growth Factor Receptor-, Cyclooxygenase-2-dependent Mechanism. *Clin Cancer Res* 2003; **9**: 2015-2021
13 **Church RD**, Fleshman JW, McLeod HL. Cyclo-oxygenase 2 inhibition in colorectal cancer therapy. *Br J Surg* 2003; **90**: 1055-1067
14 **Lim JW**, Kim H, Kim KH. Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. *Lab Invest* 2001; **81**: 349-360
15 **Maeda S**, Akanuma M, Mitsuno Y, Hirata Y, Ogura K, Yoshida H, Shiratori Y, Omata M. Distinct mechanism of *Helicobacter pylori*-mediated NF-kappa B activation between gastric cancer cells and monocytic cells. *J Biol Chem* 2001; **276**: 44856-44864
16 **Inoue H**, Nanayama T, Hara S, Yokoyama C, Tanabe T. The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells. *FEBS Lett* 1994; **350**: 51-54
17 **Ueda J**, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. *Oncogene* 2003; **22**: 8716-8722
18 **Attiga FA**, Fernandez PM, Weeraratna AT, Manyak MJ, Patierno SR. Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. *Cancer Res* 2000; **60**: 4629-4637
19 **Callejas NA**, Casado M, Diaz-Guerra MJ, Bosca L, Martin-Sanz P. Expression of cyclooxygenase-2 promotes the release of matrix metalloproteinase-2 and -9 in fetal rat hepatocytes. *Hepatology* 2001; **33**: 860-867
20 **Huls G**, Koornstra JJ, Kleibeuker JH. Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. *Lancet* 2003; **362**: 230-232
21 **Thun MJ**, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst* 2002; **94**: 252-266
22 **Chan TA**. Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol* 2002; **3**: 166-174
23 **Barnes CJ**, Cameron IL, Hardman WE, Lee M. Non-steroidol anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis. *Br J Cancer* 1998; **77**: 573-580
24 **Koga H**. Hepatocellular carcinoma: is there a potential for chemoprevention using cyclooxygenase-2 inhibitors? *Cancer* 2003; **98**: 661-667
25 **Dohadwala M**, Luo J, Zhu L, Lin Y, Dougherty GJ, Sharma S, Huang M, Pold M, Batra RK, Dubinett SM. Non-small cell lung cancer cyclooxygenase-2-dependent invasion is mediated by CD44. *J Biol Chem* 2001; **276**: 20809-20812
26 **Jiang XH**, Wong BC. Cyclooxygenase-2 inhibition and gastric cancer. *Curr Pharm Des* 2003; **9**: 2281-2288
27 **Li G**, Yang T, Yan J. Cyclooxygenase-2 increased the angiogenic and metastatic potential of tumor cells. *Biochem Biophys Res Commun* 2002; **299**: 886-890
28 **Gupta RA**, Polk DB, Krishna U, Israel DA, Yan F, DuBois RN, Peek RM Jr. Activation of peroxisome proliferator-activated receptor gamma suppresses nuclear factor kappa B-mediated apoptosis induced by *Helicobacter pylori* in gastric epithelial cells. *J Biol Chem* 2001; **276**: 31059-31066
29 **Maeda S**, Akanuma M, Mitsuno Y, Hirata Y, Ogura K, Yoshida H, Shiratori Y, Omata M. Distinct mechanism of *Helicobacter pylori*-mediated NF-kappa B activation between gastric cancer cells and monocytic cells. *J Biol Chem* 2001; **276**: 44856-44864
30 **Wada A**, Ogushi K, Kimura T, Hojo H, Mori N, Suzuki S, Kumatori A, Se M, Nakahara Y, Nakamura M, Moss J, Hirayama T. *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol* 2001; **3**: 115-123

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Bilateral induction of the S-100A9 gene in response to spreading depression is modulated by the cyclooxygenase-2 activity

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Abstract

Cyclooxygenase-2 (COX-2) was reported to be induced in the infarcted human brain. Spreading depression (SD) is thought to play a role in this induction. In this study, we correlated the expression of SD-associated genes with COX-2 production in brains after SD. Rats were divided into 3 groups: rats that did not undergo SD (group I saline controls, $n=7$), rats that underwent unilateral SD as a result of KCl application (group II, $n=9$), and rats that were pretreated with the selective COX-2 inhibitor, JTE-522 3 h before the induction of SD (group III, $n=7$). The expression of the SD-associated genes, S-100A9, and mitogen-activated proteinkinase phosphatase (cpg21) was analyzed 2 h later using a cDNA array. In group II, COX-2 and cpg21 mRNA expression, as determined by RT-PCR, were significantly upregulated in the hemisphere undergoing SD. While the expression of S-100A9 mRNA was bilaterally upregulated in these animals, this expression was significantly reduced in group III, and was accompanied by reduced bilateral production of PGE_2. Thus, the bilateral induction of expression of the S-100A9 gene in response to SD was associated with COX-2 activation.

Keywords: Spreading depression; S-100A9; cpg21; Cyclooxygenase-2; JTE-522; Rat

1. Introduction

Spreading depression (SD), characterized by reversible depression of cortical electrical activity in the brain that spreads like a wave, can be induced by a variety of electrical, chemical, and mechanical stimuli in the normal brain [1]. While preconditioning by the repeated induction of SD was shown to induce tolerance to subsequent ischemia [2], the induction of repetitive SDs in the ischemic cortex within a few hours after ischemia was found to contribute to the expansion of the infarcted areas in rat and cat models [3–6]. Thus, SD appears to have dual effects on the brain.

The activity of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin synthesis, was upregulated by SD [7] and focal brain ischemia [8] in the cortex of primates and nonprimates [9,10]. Koistinaho and Chan [11] reported that SD directly induced COX-2 expression in focal brain ischemia by stimulating the *N*-methyl-D-aspartate (NMDA) receptor and activating phospholipase A2. Nogawa et al. [12] suggested that COX-2, induced at the infarct border, might be involved in delaying neuronal death, though they did not assess COX-2 expression in the contralateral hemisphere. On the other hand, COX-2 expression was shown to be globally induced in the infarcted human brain, and delayed COX-2 induction in the hemisphere contralateral to the ischemia was speculated to play a role in promoting the remodeling of neural networks [13]. SD is thought to be involved in the induction of COX-2 in brain areas great distances from the ischemic

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regions [14], however, the mechanisms of COX-2 expression in remote brain areas from affected site have remained to be clarified.

To help clarify these mechanisms, we examined the alteration of gene expression in the whole brain under eliciting SDs after administration of a selective COX-2 inhibitor.

2. Materials and methods

2.1. SD model and brain preparation

All animal procedures were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council.

Male, Sprague–Dawley rats (240–350 g, $n=23$) were randomly divided into 3 groups. Rats in group I (control, $n=7$) were not subjected to SD, while rats in groups II ($n=9$) and III ($n=7$) were subjected to SD, with the rats in group III also receiving a selective COX-2 inhibitor JTE-522 (4-[4-cyclohexyl-2-methyloxazol-5-yl]-2-fluorobenzenesulfonamide; Central Pharmaceutical Research Institute of Japan Tobacco Inc., Osaka, Japan) 3 h prior to SD elicitation. JTE-522, which was suspended in a 0.5% carboxy methylcellulose solution and administered orally at a dose of 10 ml/kg, was reported to selectively inhibit COX-2 without affecting COX-1 [15]. All animals were anesthetized with chloral hydrate (400 mg/kg body weight i.p.) prior to the induction of SD.

SD was evoked by applying 3.3 mol/L KCl to the cortex. The anesthetized animals were mounted on a stereotaxic instrument in the prone position, with their head restrained using teeth and ear-bars. After the frontoparietal cranium was exposed by a midsagittal incision, two small burr holes were made in the right parietal skull bone and the dura carefully excised; the rostral burr hole was used to apply potassium chloride (KCl) while the more caudal hole was used to take direct current (DC) potential recordings. These two burr holes were made 7-mm apart (Fig. 1A). The DC potential was monitored with an amplifier (Iso-DAM8, World Precision Instruments, Sarasota, FL, USA) that was connected to microelectrodes (TM33B10, World Precision Instruments, Sarasota, FL, USA) that were inserted into the cortex to a depth of 1 mm. In the rats in group I, physiological saline, instead of KCl, was applied to the cortex through the rostral burr hole. Rectal temperature of all treated rats was monitored and their body temperature maintained around 37 °C with the aid of heating pads. An arterial catheter placed into the right femoral artery was used to continuously monitor heart rate and arterial pressure.

Two hours after KCl or saline application, brain tissues were perfused with cold saline and the animals were sacrificed by exsanguination under chloral hydrate anesthesia. The brains were then cut into 3 coronal sections as shown in Fig. 1. The section between slices 1 and 2 was frozen in isopentane-dry ice and stored at −80 °C for biochemical analyses.

2.2. Gene analyses

cDNA array analyses were used to search for SD-associated genes that were modulated by the administration of JTE-522. These analyses were conducted using Motorola CodeLink Bioarrays (Motorola Life Science, IL, USA), each of which contained 10,060 elements. Poly $(A)^+$ RNA extracted from the right cortices of 3 animals in each of the 3 groups (animal no 1~no 9) was pooled together (the total

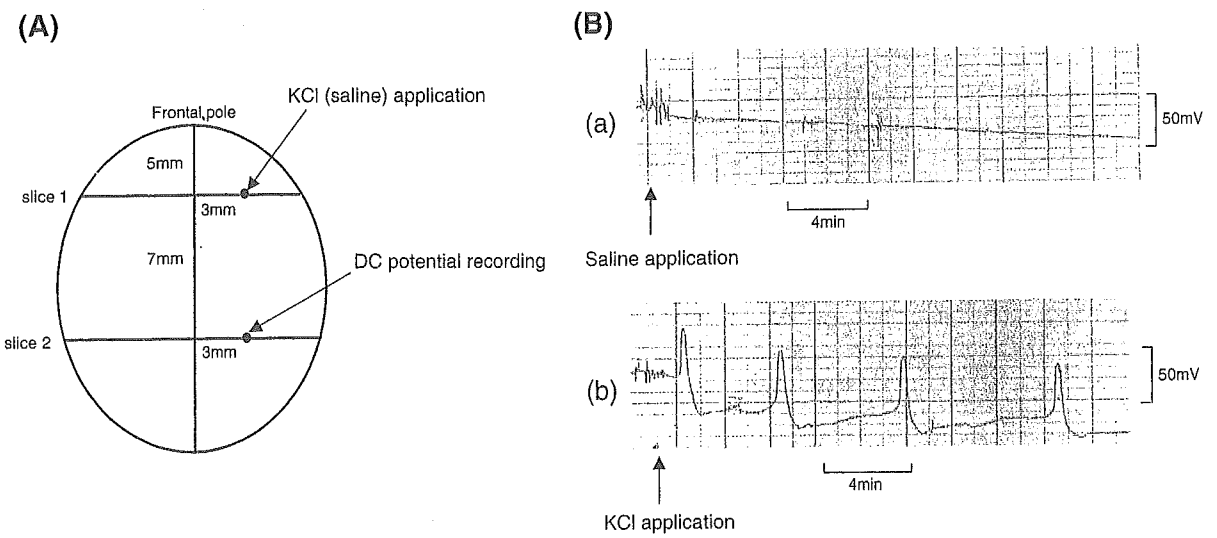

Fig. 1. Stereotaxic parameters and DC potential recordings from rats in groups I and II. (A) The brain was stereotaxically divided, on ice, into 3 coronal sections. (B) DC potentials were not detected after saline application to rats in group I (a). In the rats in group II, a total of four DC potentials were detected after KCl application (b). Tracings represent the data from a single rat in each group.

Table 1
Oligonucleotide primers used in RT-PCR

Gene	Sequences (Forward primer) (Reverse primer)	Cycling number (*N*) PCR product size
COX-2	CCCAGCACTTCACTCATCAGTTTTTCAAGA (F563)	32
	TTCCACCAGCAGGGCGGGATACAGTTCCAT (R1459)	926 bp
cpg21	GAGTATATCAAGCAGAGGAGGAGCGTGGTC (1045F)	32
	TTCCCTGAAGTGACAGAGGACAGAGACAGA (1761R)	746 bp
S-100A9	AGCGCAGCATAAGCACCATCATCAATGTTT (60F)	32
	ATTATTTCCCAGCCCCAGAACCAAGGTCAT (431R)	401 bp
GAPDH	ACCACAGTCCATGCCATCAC (586F)	23
	TCCACCACCCTGTTGCTGTA (1018R)	439 bp

COX-2, cyclooxygenase 2; cpg21, mitogen-activated protein kinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

amounted to about 30 μg) and was used to synthesize complementary DNA. The specific protocol involved the use of a Motorola CodeLink™ microarray and is described in detail elsewhere [16,17].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to examine the expression of COX-2 and SD-associated genes. Twenty samples were obtained from bilateral cortices in 10 animals (animal no10~no19). Primers for selected genes were obtained from Proligo (Kyoto, Japan). RT-PCR analysis was performed using KOD DNA polymerase (Toyobo, Osaka) as previously described [18]. The sequence of the primer pairs for each of the target genes and their cycling number (*N*) are described in Table 1. The basic cycling parameters were as follows: 3 min at 96 °C, followed by N cycles at 94 °C, 15 s; 55 °C, 2 s; 68 °C, 1 min. Cycling numbers were determined empirically using semi-quantitative PCR amplification. The amplification products were visualized by electrophoresis using a 2% ethidium bromide-stained agarose gel, and digitized using a DC290 with Kodak™ 1D 3.5.3 software. The digitized values of each gene were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4. Measurement of prostaglandin concentration by radioimmunoassay

Tissue concentrations of prostaglandin E_2 (PGE_2) in both hemispheres of all animals ($n=23$) were determined using radioimmunoassay kits (PerkinElmer Life Sciences, Inc., MA, USA). Values were normalized for protein content.

2.5. Data analysis

A one-way analysis of variance (ANOVA) followed by a Fisher's post-hoc test was used to assess the differences in hemispheric gene and PGE_2 expression between the 3 groups. A Student's *t* test was used to analyze differences between the hemispheres in each group. A two-tailed *p*-value of <0.05 was considered to be significant. Data are expressed as the mean±standard deviation.

3. Results

The physiological parameters of all test animals did not change significantly during the experimental period (Table 2). No episodes of SD were observed in the control group (I; Fig. 1). The mean number of SDs evoked by KCl application in group II (4.7±2.4) was not significantly different from that seen in group III (3.6±1.6).

3.1. cDNA array analysis

The expression signals for S-100A9 and mitogen-activated protein kinase phosphatase (cpg21) in group II increased more than 2.5-fold compared to their expression in the rats in groups I and III (2.8-fold vs. group I, 2.8-fold vs. group III for S-100A9; 6.0-fold vs. group I and 2.7-fold vs. group III for cpg21). In group II, 35 elements were found to be at least 2.5-fold different than in the rats in group I,

Table 2
Physiological measurements

Group	*N*	Weight (mg)	SD	MAP (mm Hg)	Temp. (°C)	pH	Po_2 (mm Hg)	Pco_2 (mm Hg)
I	7	302±30	0	66.4±6.5	37.2±0.6	7.30±0.02	108±15	43±4
				66.1±6.4	37.2±0.5	7.26±0.02	108±15	42±4
II	9	301±33	4.7±2.4	65.3±10.7	37.4±0.6	7.30±0.04	97±19	44±5
				68.1±12.1	37.0±0.9	7.29±0.05	108±20	42±5
III	7	305±25	3.6±1.6	74.0±9.9	37.2±0.6	7.31±0.02	104±12	44±2
				68.1±15.4	37.2±0.4	7.30±0.04	109±13	43±5

Values are the mean±S.D.
Upper rows of each column indicate baseline measurements, while the lower rows indicate values obtained 2 h after KCl or saline application.

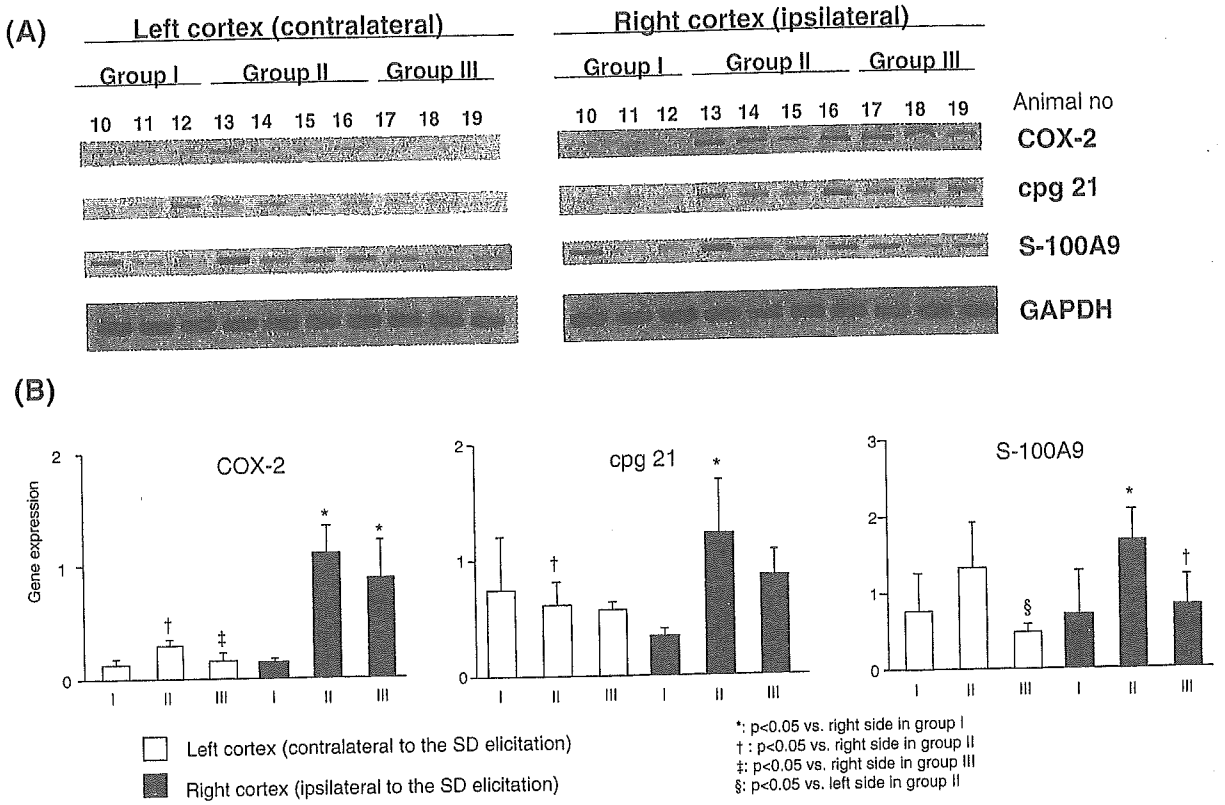

Fig. 2. RT-PCR analysis of COX-2, cpg21, S-100A9, and GAPDH mRNA in each group. (A) Autoradiograms of COX-2, cpg21, S-100A9, and GAPDH mRNA in each group. (B) Densitometric analysis showing the expression of the genes of interest normalized to GAPDH mRNA. Note that S-100A9 expression, which was upregulated in both cortices of rats in group II, was downregulated by the administration of JTE-522 (group III). The expression of COX-2 and cpg21 was prominent in the cortices undergoing SD in the rats in group II. Expression of GAPDH mRNA was equivalent between groups.

and 8 elements were found to be at least 2.5-fold different than in the rats in group III.

3.2. SD-associated genes that were modulated by JTE-522

There were no significant differences in the expression of cpg21, S-100A9, and COX-2 in the right vs. left cortex of rats in group I. However, mRNA expression of all of these genes was significantly upregulated in the right cortex of rats in group II that had undergone SD, compared to controls (Fig. 2). The expression of both COX-2 and cpg21 mRNA in the right hemisphere in group II rats was also significantly upregulated compared to their left hemisphere. On the other hand, the expression of S-100A9 mRNA in the left cortex of these rats was similar to that seen in their contralateral hemisphere. The expression of S-100A9 mRNA in both hemispheres of the rats in group III was significantly reduced compared to that seen in corresponding sides of the brain in group II rats.

Table 3
Concentration of PGE_2 in brain samples

Group	Right cortex (ipsilateral)	Left cortex (contralateral)
I	22.8±12.3	36.3±16.9
II	17.8±12.6	29.5±13.8
III	8.0±4.9***	18.4±6.9***

pg/TP (total protein) mg.
* $p<0.05$ vs. right cortex in group I.
** $p<0.05$ vs. left cortex in group III.
*** $p<0.05$ vs. left cortex in group I.

3.3. PGE_2 synthesis

Mean tissue levels of PGE_2 in both hemispheres of group III rats were significantly lower than those seen in corresponding regions of the brains of control, group I rats (Table 3). The PGE_2 concentration in the right cortex of group III rats was significantly reduced compared to levels in their contralateral hemisphere.

4. Discussion

In this study, we examined the effects of the selective COX-2 inhibitor, JTE-522, on gene expression in brains that underwent SD. We confirmed the findings of Choudhuri et al. [19] that COX-2 was only upregulated in the hemisphere that underwent SD. On the other hand, PGE_2 production levels in the rats of group II were similar to those seen in group I; levels in the right cortex of the rats in group III were significantly lower than those seen in the contralateral cortex. These findings were likely

attributable to either suppression of protein synthesis during the repetitive SDs [20], or the rapid kinetics of the COX enzyme [21,22].

cpg21, which shows 92% homology with dual specificity phosphatase 5 in humans, dephosphorylates and inactivates phosphorylated extracellular signal regulated kinase 1 (ERK1). Transient phosphorylation of ERK1/2 in a MAP kinase/ERK kinases (MEK)-dependent manner was shown to occur following SD, with phosphorylated ERK levels returning to control levels 45 min later [23]. Because JTE-522 did not suppress the expression of the cpg21 gene as shown by RT-PCR in the present study, upregulation of the cpg21 gene probably occurs concomitantly with phosphorylation of ERK1/2 after SD, and is not associated with COX-2 activation.

S-100A9 belongs to the S-100 family of calcium-binding proteins. The S-100 protein, which was first isolated from the brain by Moore in 1965 [24], exists in 3 dimeric forms [25] i.e., an alpha–alpha form known as S-100A(0), an alpha–beta form known as S-100A, and a beta–beta form known as S-100B [26]. Enhanced synthesis of S-100B by reactive astrocytes within the peri-infarct area was shown to participate in the inflammatory response that delayed infarct expansion after permanent focal ischemia in rats [27]. S-100A8 and S-100A9, which are produced by activated neutrophils and monocytes, are translocated to the cell membrane where they form a heterodimer that co-localizes with cytoskeletal proteins [28,29]. Postler et al. [30] demonstrated that microglial cells in the peri-infarct area expressed S-100A8 and S-100A9 in the early phase of human cerebral ischemia, though the role that they played in this process is not fully understood.

In the present study, we found that the expression of the S-100A9 gene was upregulated not only in the cortex that underwent SD but also in the contralateral cortex. The S-100A8/A9 complex was reported to specifically bind to polyunsaturated fatty acids in a calcium-dependent manner [31,32], and it has been implicated in the modulation of the activity of arachidonic acid-metabolizing enzymes [33]. Neuronal activity during SD that is mediated by the NMDA receptor could help propagate astrocytic calcium waves into the brain areas contralateral to the affected side [34–36]. Since S-100A9 gene expression and PGE_2 production were bilaterally downregulated in the hemispheres of rats administered JTE-522, the expression of the S-100A9 gene is likely affected not only by calcium mobilization but also by increased COX-2 activity during SD. Thus, expression of the COX-2 gene in the cortices undergoing SD could be modulated by the S100A9 gene, though it remains unclear whether the S100A9 gene could modulate contralateral COX-2 gene expression.

In conclusion, the bilateral induction of expression of the S-100A9 gene in response to SD may be modulated by prostaglandin synthesis, even though there was no upregulation of the COX-2 gene in the cortex contralateral to that which underwent SD.

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References

[1] Leao AAP. Spreading depression of activity in the cerebral cortex. J Neurophysiol 1944;7:359–90.
[2] Kobayashi S, Harris VA, Welsh FA. Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. J Cereb Blood Flow Metab 1995;15:721–7.
[3] Hossmann KA. Viability thresholds and the penumbra of focal ischemia. Ann Neurol 1994;36:557–65.
[4] Takano K, Latour LL, Formato JE, Carano RAD, Helmer KG, Hasegawa Y, et al. The role of spreading depression in focal ischemia evaluated by diffusion mapping. Ann Neurol 1996;39:308–18.
[5] Iijima T, Mies G, Hossmann KA. Repeated negative DC deflections in rat cortex following middle cerebral artery occlusion are abolished by MK-801: effect on volume of ischemic injury. J Cereb Blood Flow Metab 1992;12:727–33.
[6] Gill R, Andine P, Hillered L, Persson L, Hagberg H. The effect of MK-801 on cortical spreading depression in the penumbral zone following focal ischemia in the rat. J Cereb Blood Flow Metab 1992;12:371–9.
[7] Yokota C, Inoue H, Kuge Y, Abumiya T, Tagaya M, Hasegawa Y, et al. Cyclooxygenase-2 expression associated with spreading depression in a primate model. J Cereb Blood Flow Metab 2003;23:395–8.
[8] Yokota C, Kuge Y, Inoue H, Tagaya M, Kito G, Susumu T, et al. Post-ischemic cyclooxygenase-2 expression is regulated by the extent of cerebral blood flow reduction in non-human primates. Neurosci Lett 2003;341:37–40.
[9] Miettinen S, Fusco FR, Yrjanheikki J, Keinanen R, Hirvonen T, Roivainen R, et al. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through *N*-methyl-D-aspartic acid-receptors and phospholipase A2. Proc Natl Acad Sci USA 1997;94:6500–5.
[10] Collaco-Moraes Y, Aspey B, Harrison M, de-Belleroche J. Cyclo-oxygenase-2 messenger RNA induction in focal cerebral ischemia. J Cereb Blood Flow Metab 1996;16:1366–72.
[11] Koistinaho J, Chan PH. Spreading depression-induced cyclooxygenae-2 expression in the cortex. Neurochem Res 2000;25:645–51.
[12] Nogawa S, Zhang F, Ross ME, Iadecola C. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. J Neurosci 1997;17:2746–55.
[13] Sairanen T, Ristimaki A, Karjalainen-Lindsberg M-L, Paetau A, Kaste M, Lindsberg PJ. Cyclooxygenase-2 induced globally in infarcted human brain. Ann Neurol 1998;43:738–47.
[14] Sharp FR, Lu A, Tang Y, Millhorn DE. Multiple molecular penumbras after focal cerebral ischemia. J Cereb Blood Flow Metab 2000;20:1011–32.
[15] Matsushita M, Masaki M, Yagi Y, Tanaka T, Wakitani K. Pharmacological profile of JTE-522, novel prostaglandin H synthase-2 inhibitor, in rats. Inflamm Res 1997;46:461–6.
[16] Ramakrishnan R, Dorris D, Lublinsky A, Nguyen A, Domanus M, Prokhorova A, et al. An assessment of Motorola CodeLinkTM microarray performance for gene expression profiling applications. Nucleic Acids Research 2002;30:e30.

[17] Dorris DR, Ramakrishnan R, Trakas D, Dudzik F, Belval R, Zhao C, et al. A highly reproducible, linear, and automated sample preparation method for DNA microarrays. Genome Res 2002;12:976–84.
[18] Inoue H, Umesono K, Nishimori T, Hirata Y, Tanabe T. Glucocorticoid-mediated suppression of the promoter activity of the cyclooxygenase-2 gene is modulated by expression of its receptor in vascular endothelial cells. Biochem Biophys Res Commun 1999;254: 292–8.
[19] Choudhuri R, Cui L, Yong C, Bowyer S, Klein RM, Welch KMA, et al. Cortical spreading depression and gene regulation: relevance to migraine. Ann Neurol 2002;51:499–506.
[20] Mies G. Inhibition of protein synthesis during repetitive cortical spreading depression. J Neurochem 1993;60:360–3.
[21] Hemler ME, Lands WEM. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. J Biol Chem 1980;255:6253–61.
[22] Wu KK, Hatzakis H, Lo SS, Seong DC, Sanduja SK, Tai HH. Stimulation of de novo synthesis of prostaglandin G/H synthase in human endothelial cells by phorbol ester. J Biol Chem 1988;263: 19043–7.
[23] Chow AK, Thompson CS, Hogan MJ, Banner D, Sabourin LA, Hakim AM. Cortical spreading depression transiently activates MAP kinases. Mol Brain Res 2002;99:75–81.
[24] Moore BW. A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun 1965;19:739–44.
[25] Isobe T, Tsugira A, Okuyama T. Amino acid sequence of the subunit structure of bovine brain S-100 protein (PAP1-b). J Neurochem 1978;30:921–3.
[26] Donato R. S-100 proteins. Cell Calcium 1986;7:123–45.
[27] Matsui T, Mori T, Tateishi N, Kagamiishi Y, Satoh S, Katsube N, et al. Astrocytic activation and delayed infarct expansion after permanent focal ischemia in rats: Part I. Enhanced astrocytic synthesis of S-100beta in the periinfarct area precedes delayed infarct expansion. J Cereb Blood Flow Metab 2002;22:711–22.
[28] Roth J, Burwinkel F, van-den Bos C, Goebeler M, Vollmer E, Sorg C. MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. Blood 1993;82:1875–83.
[29] Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem 1997;272: 9496–502.
[30] Postler E, Lehr A, Schluesener H, Meyermann R. Expression of the S-100 proteins MRP-8 and-14 in ischemic brain lesions. Glia 1997;19:27–34.
[31] Siegenthaler G, Roulin K, Chatellard-Gruaz D, Hotz R, Saurat JH, Hellman U, et al. A heterocomplex formed by the calcium-binding proteins MRP8 (S100A8) and MRP14 (S100A9) binds unsaturated fatty acids with high affinity. J Biol Chem 1997;272:9371–7.
[32] Klempt M, Melkonyan H, Nacken W, Wiesmann D, Holtkemper U, Sorg C. The heterodimer of the Ca^{2+}-binding proteins MRP8 and MRP14 binds to arachidonic acid. FEBS Lett 1997;12:81–4.
[33] Kerkhoff C, Hofmann HA, Vormoor J, Melkonyan H, Roth J, Sorg C, et al. Binding of two nuclear complexes to a novel regulatory element within the human S100A9 promoter drives the S100A9 gene expression. J Biol Chem 2002;277:41879–87.
[34] Nedergaard M. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. Science 1994;25:1768–71.
[35] Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. Nature 1994;30: 744–7.
[36] Schipke CG, Boucsein C, Ohlemeyer C, Kirchhoff F, Kettenmann H. Astrocyte Ca^{2+} waves trigger responses in microglial cells in brain slices. FASEB J 2002;16:255–7.

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Nitric oxide upregulates the cyclooxygenase-2 expression through the cAMP-response element in its promoter in several cancer cell lines

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We previously showed that nitric oxide (NO) induces overexpression of cyclooxygenase-2 (COX-2) and production of prostaglandin E_2 in cancer cells. Here, we investigated the mechanisms by which NO induces COX-2 expression in cancer cells. We found that the cAMP-response element (CRE) is a critical factor in NO-induced COX-2 expression in all cells tested. We found that in cancer cells, three transcription factors (TFs) – cAMP response element-binding protein (CREB), activating transcription factor-2 (ATF-2) and c-jun, bound the CRE in the COX-2 promoter, and their activities were increased by addition of the NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). NO-induced activation of soluble guanylate cyclase (sGC), p38 and c-Jun NH_2-terminal kinase (JNK) upregulated the three TFs, leading to COX-2 overexpression. Addition of dibutyryl-cGMP (db-cGMP) induced COX-2 expression in a manner similar to SNAP; this induction was blocked by a p38 inhibitor (SB202190), but not by a JNK inhibitor (SP600125). NO-induced cGMP was found to activate CREB and ATF-2 in a p38, but not c-jun-dependent manner, while NO induced JNK in a cGMP-independent manner, leading to subsequent activation of c-jun and ATF-2. These results suggest that the low concentrations of endogenous NO present in cancer cell may induce the expression of many genes, including COX-2, which promotes the growth and survival of tumor cells.
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Introduction

In recent years, nitric oxide (NO) has been recognized as a fundamental signaling molecule for the maintenance of homeostasis, a potent cytotoxic effector molecule involved in the pathogenesis of a wide range of human diseases (Liaudet *et al.*, 2000). NO is also an important mediator of inflammatory responses, prompting researchers to investigate whether NO interacts with another inflammatory factor, cyclooxygenase-2 (COX-2), in inflammatory cells. Previous reports showed that when inducible NO synthase (iNOS) was upregulated in inflammatory cells, COX-2 expression increased in a similar pattern (Surh *et al.*, 2001). NO has been reported to activate or inhibit COX-2 activity, depending on the concentration of NO, the timing of its induction, and the investigated cell type (Salvemini *et al.*, 1993; Stadler *et al.*, 1993; Tsai *et al.*, 1994; Habib *et al.*, 1997).

It is well known that iNOS and COX-2 are simultaneously upregulated in various cancers, including head and neck cancer (Gallo *et al.*, 1998; Chan *et al.*, 1999; Son *et al.*, 2001; Lee *et al.*, 2002; Nose *et al.*, 2002). However, the direct cross-talk between NO and COX-2 in cancer cells has not been fully elucidated. Recently, we showed that NO induces COX-2 expression and prostaglandin E_2 (PGE_2) production in head and neck cancer cells, and that this effect is blocked by iNOS inhibitors. Similar results have been shown in other cancer cells, including hepatocarcinoma, gastric cancer and cervical cancer (Park *et al.*, 2003).

The role of NO in cancer is unclear and somewhat controversial, but may be divided into two broad functions: promotion of cancer progression and/or cytotoxicity to cancer cells. At high concentrations, NO acts as a tumoricidal factor, though it can also initiate tumorigenesis through genotoxic mutations. At lower (noncytotoxic) levels, NO has been reported to promote cancer progression during invasion, metastasis and angiogenesis (Gallo *et al.*, 1998; Wink *et al.*, 1998; Surh *et al.*, 2001). In cancer cells, COX-2 is known

to facilitate malignant progression though various mechanisms (Sheng *et al.*, 2001; Pai *et al.*, 2002; Sun *et al.*, 2002; Tanabe and Tohnai, 2002). COX-2 is one of the immediate early response genes; its expression is regulated by trans-activation of a regulatory signal leading to activation of specific transcription factors (TFs) that subsequently bind regulatory sites in the COX-2 promoter. NF-κB, NF-IL6, PEA-3 and cAMP response element-binding protein (CREB)/activator protein-1 (AP-1) have been suggested to play important roles in the regulation of the COX-2 expression in various cells; their response elements have been located close to the transcription initiation site of the COX-2 promoter (Howe *et al.*, 2001; Tang *et al.*, 2001; Tamura *et al.*, 2002; Tanabe and Tohnai, 2002). NO affects the activity of many TFs in various pathways. NO transduces intracellular signaling through the activation of sGC, which is the only receptor conclusively proven to mediate interactions between NO and its signaling molecules (Bogdan, 2001). However, it is not yet known how COX-2 expression is upregulated by endogenous NO in cancer cells, or whether NO plays an important role in carcinogenesis.

Here, we investigated the mechanisms of NO-induced overexpression of COX-2 by analysing the action of NO on signals related to the regulation of COX-2 expression in cancer cells. We found that NO increases COX-2 expression through the cAMP response element (CRE) of the COX-2 promoter in cancer cells. The data suggest that the low concentrations of endogenous NO present in cancer cells may induce the expressions of diverse genes, such as COX-2, which promotes the growth and survival of tumor cells.

Results

CRE is an important transcription regulatory element for basal and NO-induced COX-2 expression in SNU-1041 cells

We performed luciferase assays with plasmids containing partial COX-2 promoter sequences (−1432/+59(L), −327/+59(S), KBM, CRM, ILM, KBM+CRM, KBM+ILM, CRM+ILM and Triple M) (Figure 1b) and observed that the CRE was a critical regulatory motif for basal and NO-induced overexpression of COX-2 in SNU-1041 cells. Basal luciferase activities were significantly lower in cells transfected with plasmids containing mutant CRE (CRM, CRM+KBM, CRM+ILM and Triple M) versus those with other plasmids (−327/+59(S), KBM, ILM and KBM+ILM) (Figure 1a). Similarly, cells transfected with plasmids harboring mutant CRE sequences showed significantly lower inductions of luciferase activity in response to the NO donors, phorbol 13-myristate 12-acetate (PMA) or *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP) (Figure 1b). These data indicate that CRE is a critical regulatory motif for NO-induced COX-2 expression.

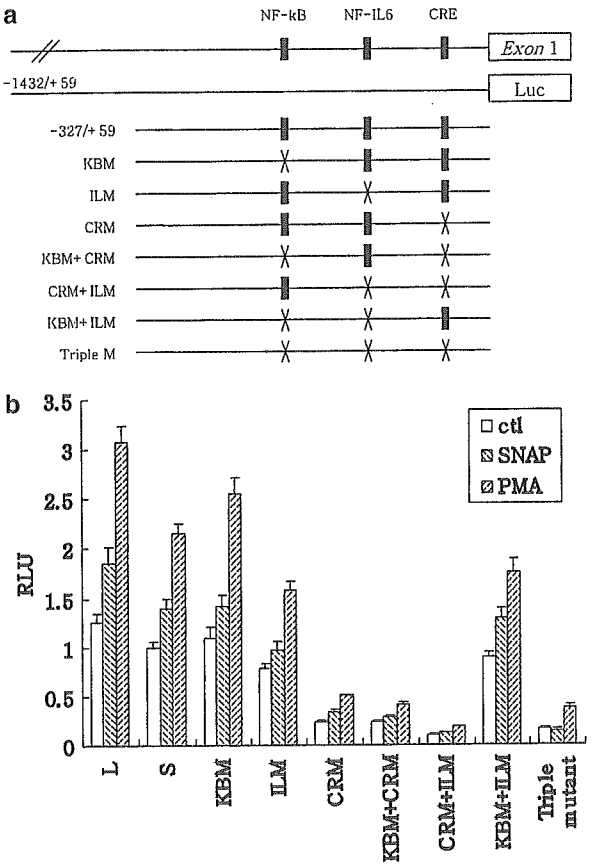

Figure 1 CRE is an important transcription regulatory element for basal and NO-induced COX-2 expression in SNU-1041 cells. (**a**) Schematic of the 5′-regulatory region of the human COX-2 gene and the utilized mutant constructs. Transcription factor binding sequences (■) and mutated sites (X) are indicated. (**b**) SNU-1041 cells were transfected with 0.5 μg of plasmids containing partial COX-2 promoters (−1432/+59(L), −327/+59(S), KBM, CRM, ILM, KBM+CRM, KBM+ILM, CRM+ILM and Triple M) and subsequently treated with 500 μM SNAP or 10 nM PMA for 18 h. Total cell lysates were prepared and COX-2 promoter activities were determined. Luciferase activity was normalized in relation to cotransfection with 0.5 μg pSV-β galactosidase control vector, and is presented in relative luciferase units (RLUs). Vertical bars indicate the standard deviation (s.d.)

CREB, activating transcription factor-2 (ATF-2) and c-jun bind to the CRE of the COX-2 promoter in SNU-1041 cells

To identify TFs that interact with the CRE of the COX-2 promoter, we performed a gel shift assay using four representative members of the CREB/ATF-2/AP-1 family of TFs: CREB, ATF-2, c-jun and c-fos (Figure 2a). Treated with antibody to CREB, ATF-2 and c-jun antibodies, but not by the anti-c-fos antibody, the decrease and dragged shift of the binding bands were detected in repeated experiments. Addition of unlabeled competitors against the CREB and AP-1 elements also decreased the intensity of the bands, while addition of an unlabeled competitor for NF-κB (cold control) did not. These results indicate that at least three of them

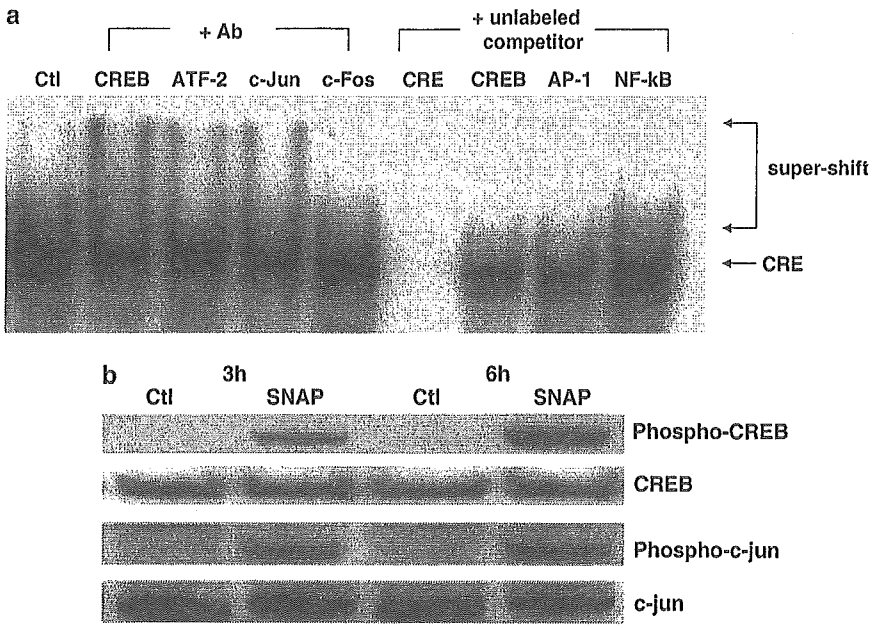

Figure 2 CREB, ATF-2 and c-jun bind to the CRE of the COX-2 promoter, and NO increases their activities in SNU-1041 cells. (**a**) Nuclear proteins (5 μg) were incubated with ^{32}P-labeled oligonucleotides containing the CRE site of COX-2, and 2 μl antibody and 1 μl unlabeled oligonucleotide competitors (25 ×) were added for competition assays. The unlabeled competitor for NF-κB was used as a cold control. (**b**) For Western blotting, cells were treated with 500 μM SNAP for 3 or 6 h. Total cellular proteins were extracted, 40 μg was loaded per lane, and the resulting immunoblots were probed for with antibodies against phospho-CREB, phospho-c-jun, CREB and c-jun

(CREB, ATF-2 and c-jun) control COX-2 expression by binding to the CRE of the COX-2 promoter.

NO phosphorylates and activates CREB, ATF-2 and c-jun in SNU-1041 cells

We next used the PathDetect Trans-Reporting system (Stratagene, La Jolla) to examine whether NO is capable of activating CREB, ATF-2 and/or c-jun. We found that NO treatment increased the activities of these TFs by 2- to 2.5-fold (Figures 4c and 5a). Western blotting revealed that CREB and c-jun were phosphorylated in cells treated with NO for 4–12 h (Figure 2b). These data indicate that NO activates the tested TFs via phosphorylation, providing a convincing link between NO and increased COX-2 expression.

NO-induced COX-2 expression is mediated partially through the cGMP-dependent pathway

As NO has been reported to control cellular signaling and gene expression through both cGMP-dependent (via activation of sGC) and -independent means (Bogdan, 2001), we examined whether cGMP is an upstream regulator of NO-induced COX-2 expression. SNU-1041 cells were treated with dibutyryl-cGMP (db-cGMP) for 18 h and their COX-2 expression was examined. The COX-2 expression in cells treated with db-cGMP was comparable to that in SNAP-treated cells (Figure 3a), and SNAP-induced COX-2 expression was inhibited by the NO-sensitive sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ)

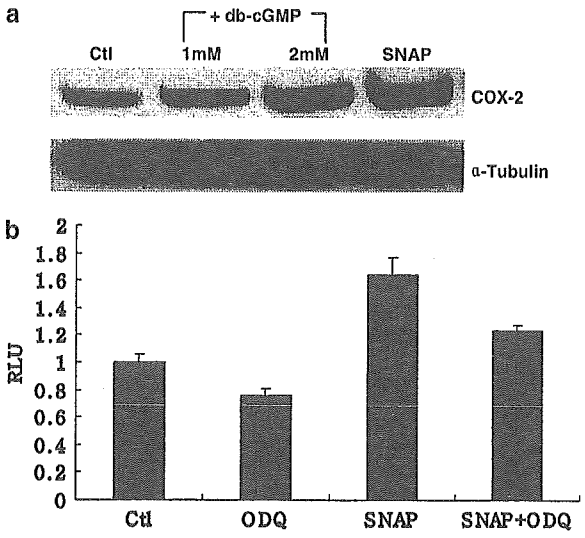

Figure 3 NO-induced COX-2 expression is mediated, in part, via the cGMP-dependent pathway in SNU-1041 cells. (**a**) Cells were treated with 1 or 2 mM dibutyryl-cGMP (db-cGMP) for 18 h and total cellular proteins (40 μg) were separated and transferred for Western blotting with an anti-COX-2 antibody. (**b**) Cells harboring the COX-2 promoter (−1432/+59) containing plasmid were incubated with 50 μM ODQ, 500 μM SNAP or 500 μM SNAP + 50 μM ODQ for 18 h and COX-2 expression was analysed in terms of luciferase activity normalized against that of β-galactosidase. The results presented are representative of three independent experiments, with the vertical bars representing the s.d.

(Figure 3b). These observations suggest that NO activates sGC to induce cGMP, leading to signals aimed at regulating COX-2 expression.

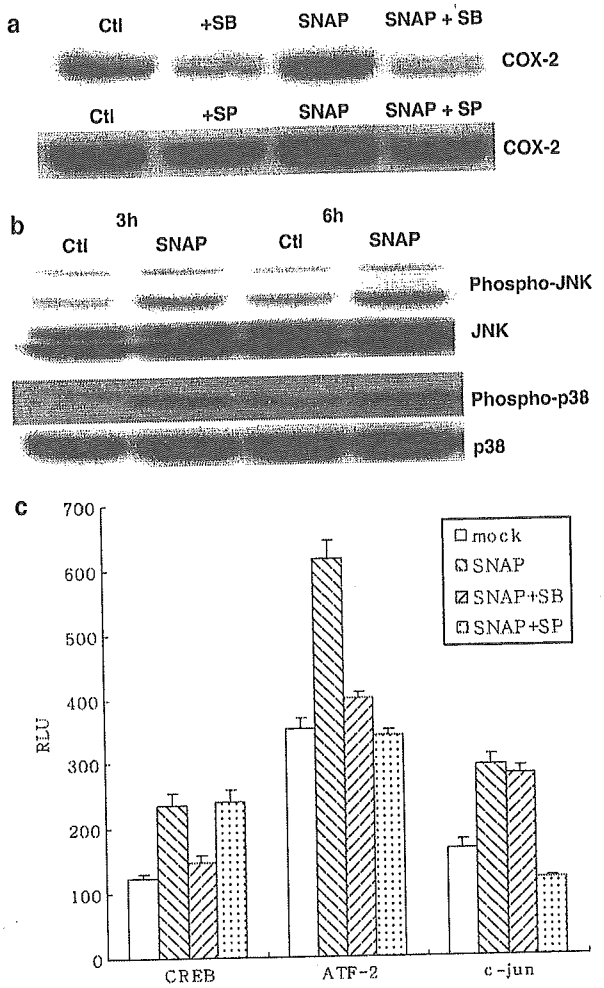

Figure 4 NO induces COX-2 expression via activation of p38 and JNK. (**a**) Cells were treated with 12.5 μM SB202190 (p38 MAPK inhibitor) or 10 μM SP600125 (JNK inhibitor) in the presence or absence of 500 μM SNAP for 18 h. Total proteins were extracted and 40 μg per lane was resolved, blotted and subjected to Western blotting with antibodies against COX-2. (**b**) Cells were treated with 500 μM SNAP for 2 or 4 h, and 40 μg of total protein was resolved and immunoblotted with antibodies against phospho-JNK, JNK, phospho-p38 and p38. (**c**) Cells transfected with 0.05 μg of pFA-ATF-2, pFA2-CREB, or pFA2-cJun were treated with 500 μM SNAP, 500 μM SNAP + 12.5 μM SB202190 or 500 μM SNAP + 10 μM SP600125 for 18 h, cell lysates were prepared, and RLUs was measured. Luciferase activity was normalized in relation to that of β-galactosidase. The vertical bars represent the s.d.

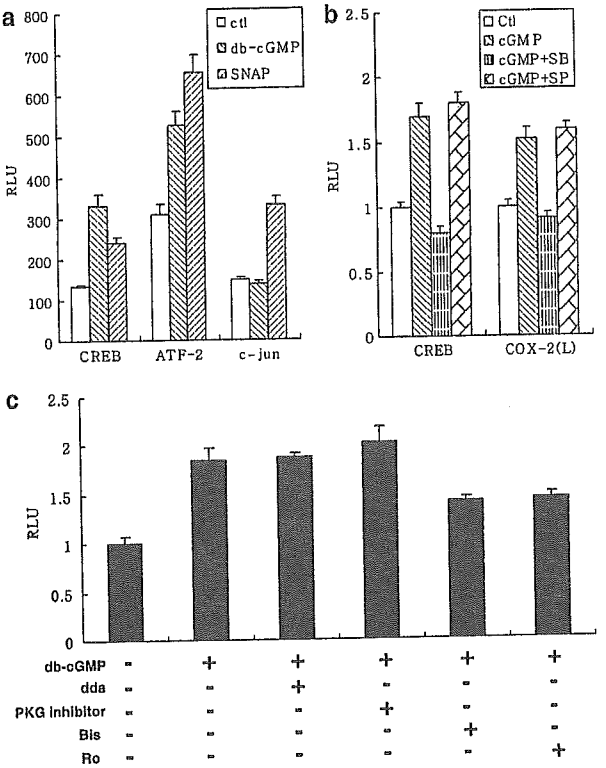

Figure 5 NO activation of p38 is cGMP-dependent, while that of JNK is cGMP-independent. Cells transfected with the indicated plasmids were treated for 18 h with (**a**) 2 mM db-cGMP or 500 μM SNAP, or (**b**) 2 mM db-cGMP, 2 mM db-cGMP + 12.5 μM SB202190, or 2 mM db-cGMP + 10 μM SP600125. (**c**) Cells transfected with pFA2-CREB were treated for 18 h with 2 mM db-cGMP, 2 mM db-cGMP + 200 μM 2-3-dda, 2 mM db-cGMP + 200 μM Rp-8-pCPT-cGMPs, 2 mM db-cGMP + 2 μM Bi-sindolylmaleimide I or 2 mM db-cGMP + 1 μM Ro-31-8220. Total cell lysates were extracted and RLUs were measured. Luciferase activities were normalized to that of β-galactosidase

NO induces COX-2 expression via activation of p38 and c-Jun NH_2-terminal kinase (JNK)

As three MAP kinases were known to be upstream regulators of COX-2 expression, we examined whether extracellular signal-regulated kinase (ERK), p38 and JNK were related to NO-induced COX-2 expression. NO-induced COX-2 expression was blocked by the p38 inhibitor, SB202190, and the JNK inhibitor, SP600125 (Figure 4a), but not by the ERK inhibitors, U0126 and PD153015 (data not shown). In addition, Western blotting revealed that the phosphorylation levels of p38 and JNK increased after 2–8 h of treatment with SNAP (Figure 4b), indicating that NO increased the phosphorylation of p38 and JNK. Furthermore, SB202190 treatment inhibited the NO-induced activation of CREB and ATF-2, while SP600125 treatment inhibited the NO-induced activation of ATF-2 and c-jun. These data indicate that among the CRE-associated TFs in the COX-2 promoter, CREB is activated by p38, c-jun is activated by JNK, and ATF-2 is activated by both (Figure 4c).

NO-induced activation of p38 is cGMP-dependent, while that of JNK is cGMP-independent

As we found that cGMP and two MAP kinases mediated NO-induced COX-2 expression, we examined whether there was any interaction between cGMP and MAP kinases in NO-induced COX-2 expression. Treatment of cells with db-cGMP upregulated the activity of

the p38 targets – CREB and ATF-2 – but not that of the JNK target – c-jun (Figure 5a). The db-cGMP-induced activities of CREB and the COX-2 promoter were blocked by the p38 inhibitor but not by the JNK inhibitor (Figure 5b). These data suggest that NO-induced cGMP upregulates p38 MAP kinase but not JNK. In an effort to identify the cGMP-activated upstream regulators of p38, we tested the effect of PKA, protein kinase-C (PKC), and protein kinase G (PKG) on cGMP-induced p38 activation. The PKC inhibitors (Bisindolylmaleimide I and Ro-31-8220) reduced the activation of p38 by ~30%, while inhibitors of PKA, PKG and Ca^{2+} had no detectable effects on the activation of p38 (Figure 5c). Thus, while we were unable to specifically define the regulators upstream of cGMP in this pathway, our results suggest some possible involvement of PKC.

The effect of NOS inhibitors on the activities of CREB and c-jun in SNU-1041 cells

To examine the effect of endogenous NO on the intracellular signaling pathways in cancer cells, we treated SNU-1041 cells with the universal NOS inhibitor, *N*-nitro-L-arginine-methyl ester (L-NAME), and the iNOS-specific inhibitors, 1400W and Aminoguanidine (AG), and examined their effects on the activities of CREB and c-jun. Treatment of cells with the NOS inhibitors inhibited the activities of CREB and c-jun (Figure 6). As NO-induced activation of these TFs is critical to the upregulation of COX-2 expression, these results suggest that COX-2 expression is likely to be decreased by blockage of NO production.

The effect of NO on COX-2 expression in other cancer cells

We previously observed that NO induces COX-2 overexpression in various cancer cells (Park *et al.*, 2003). To

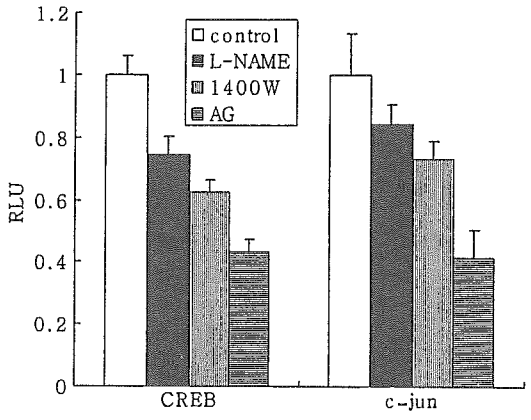

Figure 6 The effect of NOS inhibitors on the activity of CREB and c-jun in SNU-1041 cells. Cells transfected with the indicated plasmids were treated for 18 h with 1 mM L-NAME, 100 μM 1400W or 2 mM Aminoguanidine. Total cell lysates were extracted and the RLUs were measured. Luciferase activities were normalized to that of β-galactosidase

investigate whether the NO-induced responses identified in this study are seen in cancer cells other than SNU-1041, we tested the effect of NO on another head and neck cancer cell line (PCI-13) and two cell lines originating from other cancer types (SNU-668 and HeLa). In PCI-13 and HeLa cells, the CRE was important for NO-induced COX-2 expression (Figure 7a), and this increase in COX-2 expression was blocked by the p38 inhibitor (Figure 7b and c). Furthermore, NO treatment upregulated the p38 targets, CREB and ATF-2, in these cells (Figure 7d, e). In SNU-668, COX-2 expression was increased by NO treatment, and this effect was partially reduced by p38 and JNK inhibitors (Figure 7c). Collectively, these observations indicate that NO upregulates COX-2 expression by activating p38 and/or JNK in diverse cancer cells.

Discussion

We previously reported that NO induces COX-2 expression in cancer cells (Park *et al.*, 2003). Other groups have noted that while iNOS and COX-2 are barely detectable in resting inflammatory cells, their expression levels are transiently upregulated in response to signals such as growth factors and stress (Posadas *et al.*, 2000; Surh *et al.*, 2001). However, we have observed that iNOS and COX-2 are constitutively overexpressed in all tested head and neck squamous cell carcinoma (HNSCC) cell lines, prompting us to question whether the regulatory mechanisms of their expression differ in inflammatory and cancer cells.

It has been well established that NF-κB, NF-IL6, PEA-3 and CREB/AP-1 are the main TFs responsible for the regulation of COX-2 expression (Howe *et al.*, 2001; Tang *et al.*, 2001; Tamura *et al.*, 2002; Tanabe and Tohnai, 2002). In addition, a growing body of evidence indicates that NF-κB plays a central role in the general inflammatory response and may be important in the upregulation of COX-2 expression in macrophage-like cells (Chabot-Fletcher, 1996). NF-κB has been suggested to play a role in the regulation of COX-2 expression in cancer cells (Luque and Gelinas, 1997; Surh *et al.*, 2001), but CRE-binding factors have been more frequently associated with COX-2 expression in these cells (Xu *et al.*, 1997; Guo *et al.*, 2001; Subbaramaiah *et al.*, 2002). In this study, we observed that the CRE element is critical for activation of the COX-2 promoter in all cells tested. Furthermore, a mutation in the NF-κB binding site had no effect on COX-2 expression, and the NF-κB inhibitors tested in this work had no affect on expression of COX-2 (data not shown). These observations suggest that differential COX-2 expression in cancer cells may depend on epigenetic events within the regulatory region of genes or on intracellular signaling generated during carcinogenesis (Song *et al.*, 2001).

We investigated whether the NO-induced overexpression of COX-2 in cancer cells was mediated by CRE

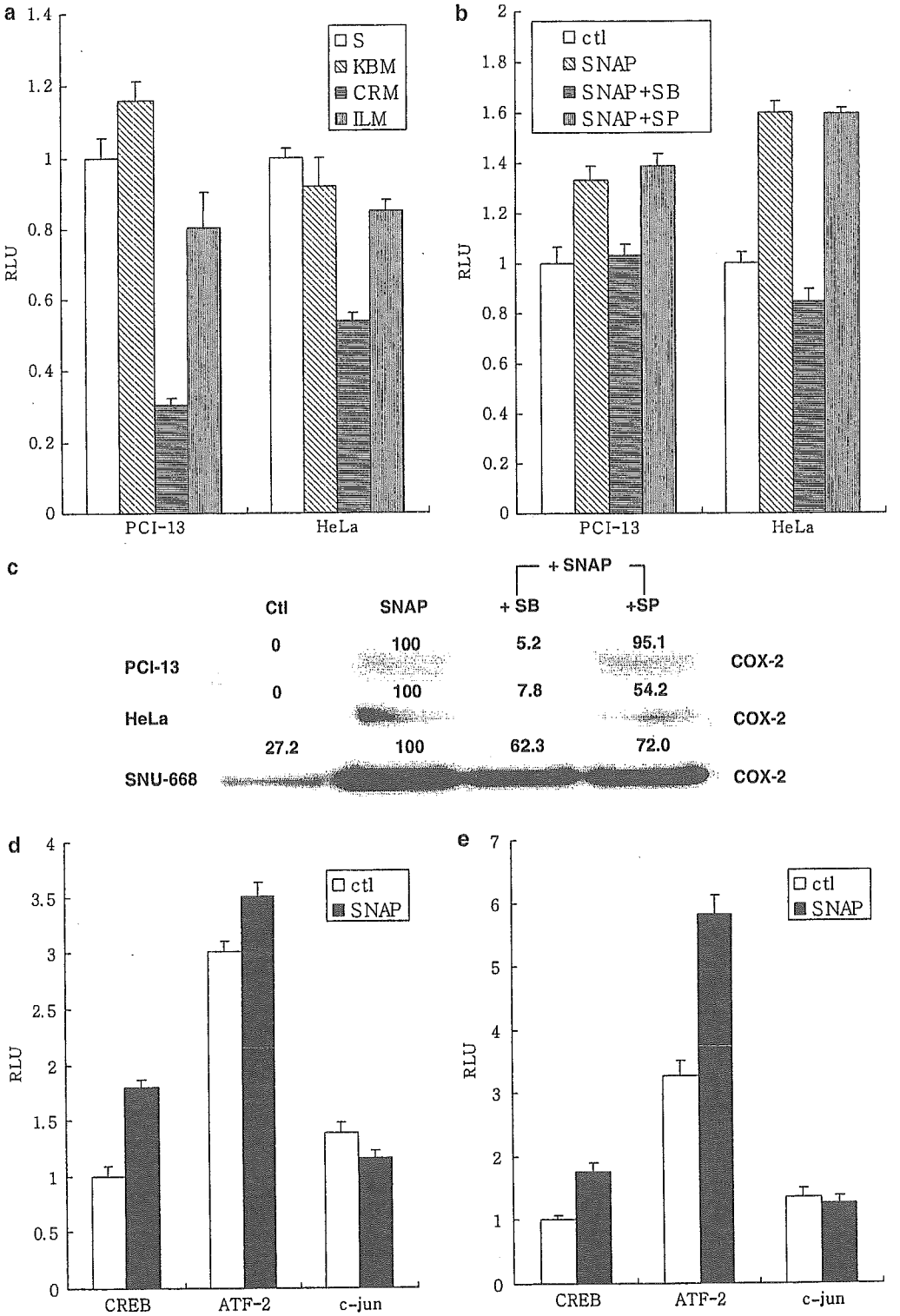

Figure 7 The effect of NO on COX-2 expression in other cancer cells. (**a**) The COX-2 promoter activity was measured in PCI-13 and HeLa cells transfected with a partial COX-2 promoter (−327/+59(S), KBM, CRM or ILM). (**b**) PCI-13 or HeLa cells transfected with the partial COX-2 promoter (−1432/+59) were treated for 18 h with 500 μM SNAP, 500 μM SNAP + 12.5 μM SB202190 or 500 μM SNAP + 10 μM SP6001125. Cell extracts were screened for COX-2 promoter activity. (**c**) Un-transfected PCI-13, HeLa and SNU-668 cells were treated for 18 h with 500 μM SNAP, 500 μM SNAP + 12.5 μM SB202190 or 500 μM SNAP + 10 μM SP6001125. Cell extracts (40 μg of total protein) were used for Western blotting. The variation of COX-2 expression by p38 and JNK inhibitors was analysed quantitatively by densitometry and indicated in figure. (**d**, **e**) SNAP (500 μM) was added for 18 h to (**d**) PCI-13 and (**e**) HeLa cells transfected with pFA-ATF-2, pFA2-CREB or pFA2-cJun. Cell lysates were used to determine the activity of each transcription factor

element-interacting factors. AP-1 family members bind to the CREB site in many cells (Park *et al.*, 1999). Although the CRE sequence in the COX-2 promoter (5′-TTCGTCA-3′) differs slightly from the consensus sequences for CREB (5′-TGACGTCA-3′) and AP-1 (5′-TGAGTCA-3′), studies have shown that CREB/ATF-2 and AP-1 interact with the CRE of COX-2 (Xu *et al.*, 1997; Guo *et al.*, 2001). Similarly, we observed that CREB, ATF-2 and c-jun, but not c-fos, bind to the CRE of the COX-2 promoter in the tested cells. In addition, we showed that NO activates CREB, ATF-2 and c-jun. However, the NO-induced activations of these factors appear to be regulated by different mechanisms. Phosphorylation of CREB was increased by the NO-induced activation of sGC, which subsequently activated p38 mitogen-activated protein kinase (MAPK), an upstream regulator of CREB. In contrast, c-jun was phosphorylated by the direct activation of JNK by NO, and ATF-2 activity was upregulated by both p38 and JNK (Figure 8). We were unable to identify the specific molecule linking cGMP signaling to activation of p38, but did observe that inhibition of PKC reduced p38 activation by ~30%. Previous studies reported that p38 could be activated by PKG via an unknown mechanism (Browning *et al.*, 2000). However, PKA, PKG and Ca^{2+} did not appear to be involved in NO-induced upregulation of p38, suggesting that one or more other regulator(s) may participate in the activation of p38 by cGMP in our model.

Our results indicate that NO appears to upregulate various signaling molecules, leading to activation of TFs related to CRE of COX-2 promoter and increased expression of COX-2 in cancer cell lines derived from HNSCC (SNU-1041, PCI-13), cervical cancer (HeLa), and gastric cancer (SNU-668) tissues. In addition, another group has shown that the effect of NO on PEA-3-mediated induction of COX-2 is relevant in colorectal cancer cells (Liu *et al.*, 2004). This means that NO might affect COX-2 expression in various mechanisms.

Other potential upstream regulators of COX-2 overexpression in cancer cells reportedly include epidermal growth factor (EGF) receptor activation (Coffery *et al.*, 1997), Her-2/neu activation (Vadlamudi *et al.*, 1999), p53 mutation (Han *et al.*, 2002) and ras mutation (Slice *et al.*, 2000). Interestingly, Her-2/neu activation and ras mutation both lead activation of the CRE element in the COX-2 promoter (Slice *et al.*, 2000; Subbaramaiah *et al.*, 2002).

Unlike lipopolysaccharide (LPS), EGF and PMA, which strongly induce COX-2, NO is a relatively weak inducer of COX-2 expression. While these strong inducers are capable of triggering increased COX-2 expression even in cells with low basal COX-2 expression, our data indicate that NO was only able to efficiently induce COX-2 expression in cells with moderate to strong basal COX-2 expression. We thus postulate that in cancer cells, the COX-2-inducible signaling molecules are constitutively activated such that they are sensitive to further activation by NO. In noncancerous cells, however, the NO-responsive molecules are only weakly activated and are unable to respond to NO. Thus, NO may act to accelerate existing intracellular signals rather than generating new signals.

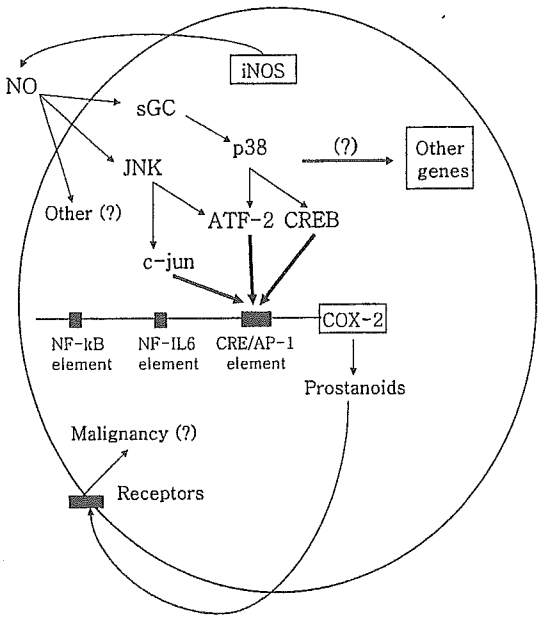

Figure 8 Possible mechanisms of NO-derived signal contributions to COX-2 expression in cancer cells. Endogenous or abnormally induced NO in the cancer microenvironment may upregulate COX-2 expression via activation of the transcription factors that associate with the CRE of the COX-2 promoter. Our data seem to suggest that NO may accelerate existing intracellular signals, rather than generating new signaling. We thus postulate that in cancer cells, NO is able to upregulate the expression of COX-2 because COX-2-inducible signaling molecules are already constitutively activated, leaving them sensitized to NO signaling. In noncancer cells, however, the NO-responsive molecules are only weakly activated, so NO is unable to induce COX-2 expression. In this way, NO may induce the expression of COX-2 and possibly other factors that promote the growth and survival of tumor cells

COX-2 is thought to play an important role in carcinogenesis, suggesting that its blockade could be beneficial in terms of cancer management. However, regulation of COX-2 expression is a complex process mediated by numerous factors, complicating efforts to clinically block COX-2 expression. Moreover, the currently available COX-2 inhibitors block both COX-1 and COX-2 in normal cells during inflammatory responses, limiting their applicability to cancer therapy. Thus, it is important to identify differences in regulation of COX-2 expression between cancerous and noncancerous cells, as these differences could allow targeting of the cancer-specific COX-2 pathway(s) for novel therapeutic strategies.

In conclusion, our data suggest that the cAMP-response element is a key regulator of COX-2 expression in cancer cells (i.e. head and neck cancer cells), and that endogenous or abnormally induced NO in the cancer microenvironment may upregulate COX-2 expression through the activation of TFs capable of binding the